

Increased *N*-acylphosphatidylethanolamine biosynthesis in elicitor-treated tobacco cells

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Previous studies with tobacco (*Nicotiana tabacum* L.) cell suspensions indicated that elicitation of defense response (production of phytoalexins) with xylanase (1,4- β -D-xylanxylanohydrolase; EC 3.2.1.8) resulted in a dramatic acylation of phytosterols (Moreau et al. 1994). *N*-acylphosphatidylethanolamine (NAPE), an acylated derivative of phosphatidylethanolamine (PE), was recently demonstrated to be synthesized in vivo in plant tissues (Chapman and Moore 1993a). Here we report that acylation of PE was increased in elicitor-treated cells. NAPE levels increased 3-fold (from 1.6 to 4.8 mol% of total phospholipids) after a 2-h treatment of cell suspensions with xylanase (1 μ g ml⁻¹). Specific activity of NAPE synthase increased in parallel with NAPE levels. Levels of NAPE and NAPE synthase activity declined during the period of 2–4 h after elicitation while levels of acylated sterolglycosides (ASG) continued to increase. Radiolabeling studies with [2-¹⁴C]-ethanolamine confirmed that three times as much NAPE was synthesized in elicitor-treated cells compared to that in unelicited cells. Patterns of incorporation of [1-¹⁴C]-palmitic acid into membrane phospholipids in elicitor-treated cells suggested that increased acylation of lipids may be a result of changes in the acyl-coenzyme A pool. Treatment of cells with purified ethylene biosynthesis-inducing xylanase (EIX; 1 μ g ml⁻¹ cells) resulted in increased levels of NAPE synthase activity comparable to those observed with the commercial preparations of xylanase. Boiled xylanase did not elicit an increase in the specific activity of NAPE synthase. Collectively our results demonstrate that the accumulation of NAPE in tobacco cells is attributable to increased activity of NAPE synthase. This suggests that NAPE may be specifically synthesized to play a protective role in membranes of plant cells as has been suggested for membranes of damaged animal cells.

Key words – Acyltransferases, cell cultures, elicitor, ethylene biosynthesis-inducing xylanase, fatty acids, lipid metabolism, *Nicotiana tabacum*, phosphatidylethanolamine, phospholipids, signal transduction, sterols, sterol glycosides, tobacco, xylanase.

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Introduction

Several groups (Anderson et al. 1990, Chappell et al. 1987, Felix et al. 1993, Lotan and Fluhr 1990, Moreau et al. 1994, Tanaka and Fujimori 1985, Threlfall and Whitehead 1988) have identified xylanase as the active molecular component of fungal pathogens effecting various defense responses (including ethylene biosynthesis, production of phytoalexins, synthesis of patho-

genesis-related proteins, etc.) in plant cells and tissues. For example, purified xylanase (1 g ml⁻¹) from *Trichoderma viride* elicited the biosynthesis and secretion of sesquiterpene phytoalexins (Moreau et al. 1994) in tobacco cell suspension cultures, a response previously characterized with crude fungal cell walls and/or enzyme preparations (Chappell et al. 1987, Moreau and Preisig 1993).

In efforts to uncover the mechanism(s) of signal trans-

duction in plant pathogen perception. Moreau and co-workers (Moreau and Preisig 1993, Moreau et al. 1994) demonstrated that detectable changes in membrane lipid composition preceded the accumulation of phytoalexins. A dramatic increase in the fatty acylation of free sterols (St) and sterol glycosides (SG) was reported after a 4-h treatment with xylanase, while the accumulation of extracellular phytoalexins was detectable only after 8 h treatment with xylanase. Because St, SG and acylated SG (ASG) are believed to be primarily confined to plasma membranes of plant cells (Lynch and Steponkus 1987), changes in the relative proportions of these lipids could be interpreted to indicate a role in plant defense at the plasma membrane level. In addition to *Trichoderma viride* xylanase, treatment with a variety of crude (cellulases) and purified (ethylene biosynthesis-inducing xylanase, EIX) fungal enzymes as well as metal ions resulted in an increase in sterol esters (StE) and ASG, suggesting that the acylation of phytosterols is a common response to several perceived stresses.

Acylation of St and SG prompted us to investigate the possible involvement of the acylation phenomenon recently reported to occur on the membrane phospholipid, phosphatidylethanolamine (PE). *N*-acylphosphatidylethanolamine (NAPE) was shown to be a widespread, albeit minor, phospholipid component in membranes of plant cells (Chapman and Moore 1993a). NAPE was synthesized in cotyledons of cotton seedlings during postgerminative growth by a direct acylation of the ethanolamine head group of PE (Chapman and Moore 1993a) with free fatty acids (FFA) (Chapman and Moore 1993b).

In animal tissues, NAPE accumulation was associated with tissue damage (e.g. myocardial infarction, brain ischemia, etc.; reviewed in Schmid et al. 1990). Biophysical studies with aqueous dispersions of NAPE were interpreted to indicate that this lipid acts to stabilize bilayer configurations of lipids (Akoka et al. 1988, Lafrance et al. 1990) suggesting that NAPE may play a protective role by preserving membrane structure. In the present study, NAPE biosynthesis in tobacco cell suspensions was increased in the presence of xylanase as judged by quantification of NAPE levels, NAPE synthase enzyme activity measurements, and radiolabeling experiments *in vivo* with [2-¹⁴C]-ethanolamine. These results extend previous observations of lipid changes that occur in plant cells during elicitation of defense responses to include acylation of PE. Perhaps accumulation of NAPE in plant cells is associated with membrane protection, as was previously suggested for animal cells (Schmid et al. 1990).

Abbreviations – ASG, acylated sterolglycoside(s); EIX, ethylene biosynthesis-inducing xylanase; FFA, free fatty acid(s); LPC, lysophosphatidylcholine(s); NAPE, *N*-acylphosphatidylethanolamine(s); PE, phosphatidylethanolamine(s); PC, phosphatidylcholine(s); SG, sterol glycoside(s); St, free sterol(s), StE, sterol ester(s).

Materials and methods

Cell cultures and elicitation

Nicotiana tabacum KY 14 cell suspension cultures were originally obtained from Dr Joseph Chappell of the University of Kentucky, Lexington, KY, USA, and maintained at 25°C on modified Murashige-Skoog medium as previously described (Chappell et al. 1987). Cells were subcultured by three-fold dilution with fresh medium every 7 days; 3-day-old cultures were treated with xylanase (1,4-β-D-xylanxylanohydrolase) (1 μg [ml cells]⁻¹) from *Trichoderma viride* (Sigma) for elicitation experiments as previously developed (Moreau et al. 1994). This commercial preparation of xylanase is reported by Sigma to contain <0.02% cellulase, <0.01% β-glucosidase and 0.002% β-xylosidase activities. For some experiments, cells were treated with purified ethylene biosynthesis-inducing xylanase (EIX, 1 μg [ml cells]⁻¹) which was kindly provided to us by Dr James D. Anderson of the USDA-ARS Weed Science Laboratory, Beltsville, MD, USA. The EIX was purified from *Trichoderma viride* to electrophoretic homogeneity as judged by sodium dodecyl sulphate polyacrylamide gel electrophoresis (Dean and Anderson 1991).

Enzyme activity

Cells were routinely collected by vacuum filtration, washed with 25 ml fresh culture medium, and weighed. Typically 4–6 g of cells were present in each 125-ml flask after 3 days of growth. Cells were homogenized in a chilled mortar by grinding cells with a pestle in an equal volume of 100 mM potassium phosphate buffer (pH 7.2), containing 10 mM KCl, 1 mM ethylene glycol-bis(β-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM MgCl₂ and 400 mM sucrose. NAPE synthase activity was measured directly in homogenates by following the incorporation of [1-¹⁴C]-palmitic acid into phosphatidyl-(*N*-palmitoyl)ethanolamine as previously described (Chapman and Moore 1993b, McAndrew et al. 1995). Protein was quantified by a modification of the method of Bradford (Stoscheck 1990) using bovine serum albumin (BioRad, Inc., Hercules, CA, USA) as the standard.

Spectroscopy

Absorption spectra were recorded on a Milton Roy (Rochester, NY, USA) Genesys5 spectrophotometer. A compound(s) with absorption maximum at 382 nm that increased with time of elicitation was identified in homogenates of elicited cells by using homogenates of unelicited cells as a blank. Thereafter, absorbance at 382 nm was measured directly on homogenates as an indicator of effective elicitation.

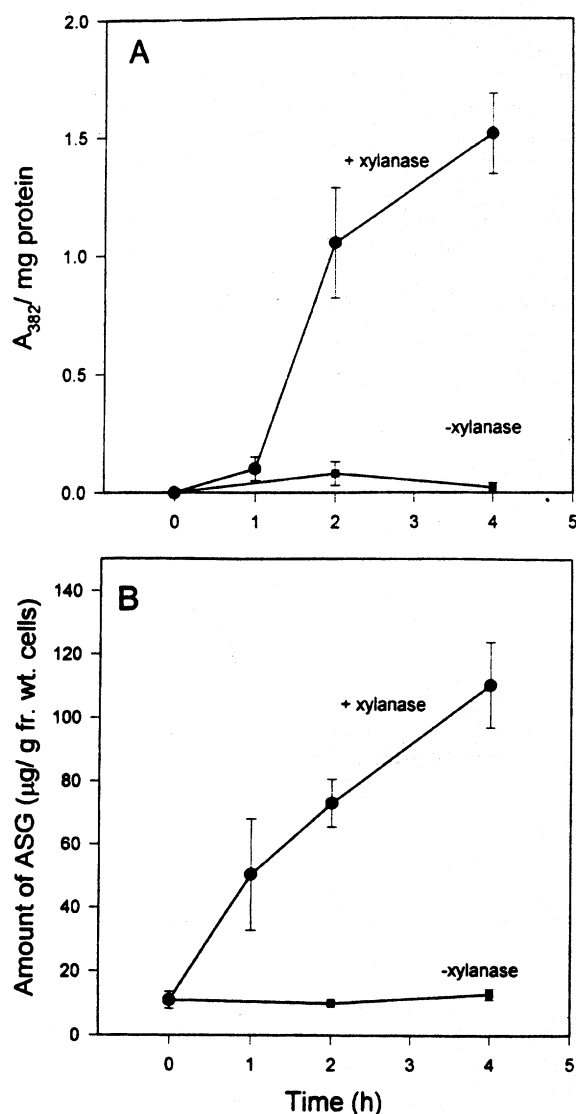


Fig. 1. Changes in levels of A₃₈₂-absorbing compound(s) in cell homogenates (panel A) and ASG (panel B) in unelicited (squares) and elicitor-treated (circles) tobacco cell suspensions. Data points are the mean and SD of triplicate samples from a representative experiment. Similar trends were observed in three replicate experiments.

Lipid analyses

For lipid extractions, cells were powdered in liquid N₂ in a chilled mortar, added to boiling isopropanol (in a ratio of 0.8 g powdered cells/2 ml isopropanol) and heated (70°C) for 30 min as a precaution to inactivate endogenous phospholipases. Chloroform was added (1 ml chloroform per 2 ml isopropanol) and lipids were extracted overnight at 4°C then washed as previously described (Chapman and Moore 1993a).

ASG levels were quantified in total lipid extracts by high performance liquid chromatography coupled with

flame ionization detection (HPLC-FID) as previously described (Moreau et al. 1990). Phospholipid phosphate was determined by the method of Duck-chong (1979) following digestion in magnesium nitrate and was expressed in mol.

Radiolabeling

To specifically examine the biosynthesis of ethanolamine-containing phospholipids during elicitation, cultures were incubated with [2-¹⁴C]-ethanolamine (370 kBq; 111 GBq mol⁻¹, ICN Biomedicals, Inc., Costa Mesa, CA, USA) for 4 h with or without xylanase. Lipids were extracted as described above. Polar lipids were purified with silica gel SPE cartridges (Whatman, Inc., Clifton, NJ, USA), separated by two-dimensional thin layer chromatography (TLC), and subjected to autoradiography as previously described (Chapman and Moore 1993a). Co-chromatography of radiolabeled tobacco NAPE with synthetic NAPE (dipalmitoyl phosphatidyl-[N-palmitoyl] ethanolamine) and radiolabeled cottonseed NAPE (see Chapman and Moore 1993a) helped to confirm its identification. Radiolabeled phospholipids were quantified by liquid scintillation counting (Beckman Instruments model LS 8000, Fullerton, CA, USA).

Results

Tobacco cells visibly browned after 2 h treatment with xylanase. This fact led us to believe that the effectiveness of elicitation treatments might be quickly evaluated by UV-visible spectroscopy of cell homogenates. A₃₈₂-

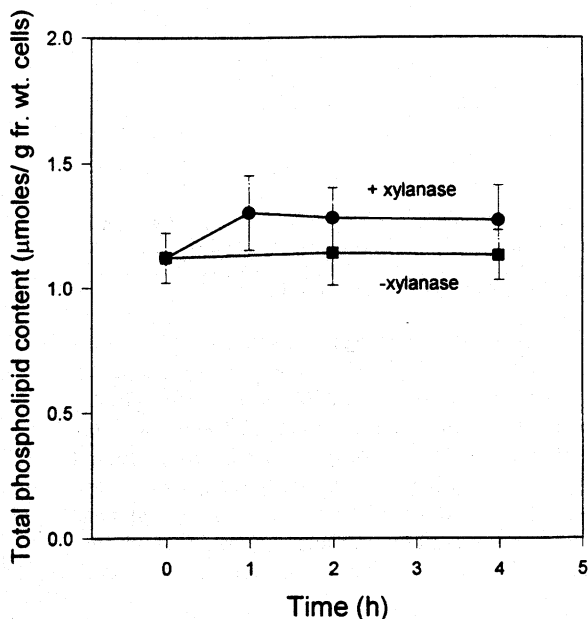


Fig. 2. Total phospholipid content in unelicited (squares) and elicitor-treated (circles) tobacco cell suspensions. Data points are the mean and SE of three independent elicitation experiments.

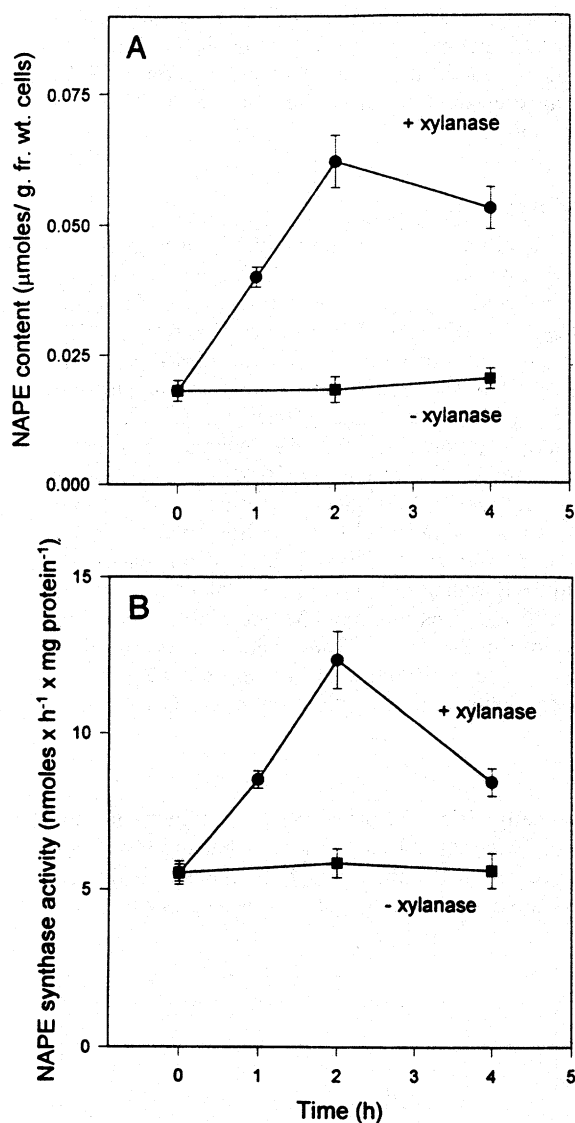


Fig. 3. Changes in the levels of NAPE (panel A) and NAPE synthase specific activity in unelicited (squares) and elicitor-treated (circles) tobacco cell suspensions. Data points are the mean and SE of three independent elicitation experiments.

absorbing material was present in elicited cells (compared to unelicited cells) and increased with increasing time of exposure to xylanase (Fig. 1A). Hence, elicitation treatments were routinely evaluated and standardized by UV-visible spectroscopy. ASG levels increased linearly with increasing time of xylanase exposure to approximately 10-fold after 4 h incubation in xylanase (Fig. 1B), verifying that increasing absorption at 382 nm (Fig. 1A) was correlated with this documented elicitation response.

Total phospholipid content was not significantly different in elicited cells compared to that of unelicited cells (Fig. 2). Total protein content in cell homogenates averaged approximately 62 mg (g fresh weight)⁻¹ and

was not significantly different in elicited cells compared to that of unelicited cells (not shown).

The amount of NAPE increased three-fold during the first 2 h of elicitation with xylanase (from 0.018 mol to 0.062 mol [g fresh weight]⁻¹) and then declined slightly (to 0.053 mol [g fresh weight]⁻¹) in the 4-h sample (Fig. 3A). Normal levels of NAPE in unelicited cells were 1.6 mol% of total phospholipid. Maximum levels of NAPE in elicited cells reached 4.8 mol% of total phospholipid.

A transient increase in specific activity of NAPE synthase was observed upon treatment of cells with xyla-

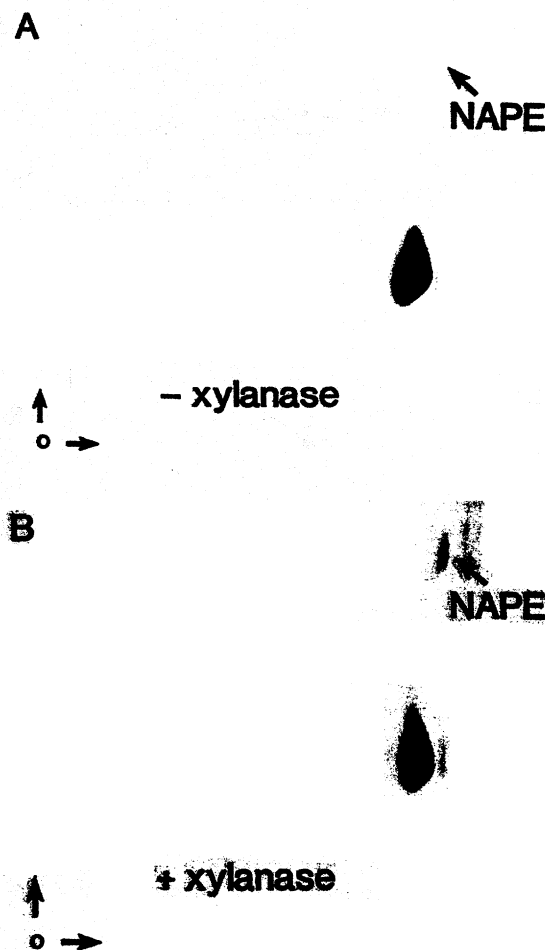


Fig. 4. Autoradiograms of two-dimensional TLC separations of phospholipids extracted from unelicited (panel A) and elicitor-treated (panel B) tobacco cell suspensions incubated with [2-¹⁴C]-ethanolamine for 4 h. Approximately 20 000 dpm were separated on each plate (silica gel G-60, 0.25 mm layer, 20 × 20 cm). The circle in the lower left corner marks the origin and the arrows indicate the directions of solvent development (first, toward the top in chloroform/methanol/7 M NH₄OH, 65:30:4, and then to the right in chloroform/methanol/acetic acid/water, 85:12.5:12.5:3). Exposure of TLC plates to X-ray film (Kodak XAR film) was for 7 days at room temperature. The position of radiolabeled NAPE is marked in the upper right corner.

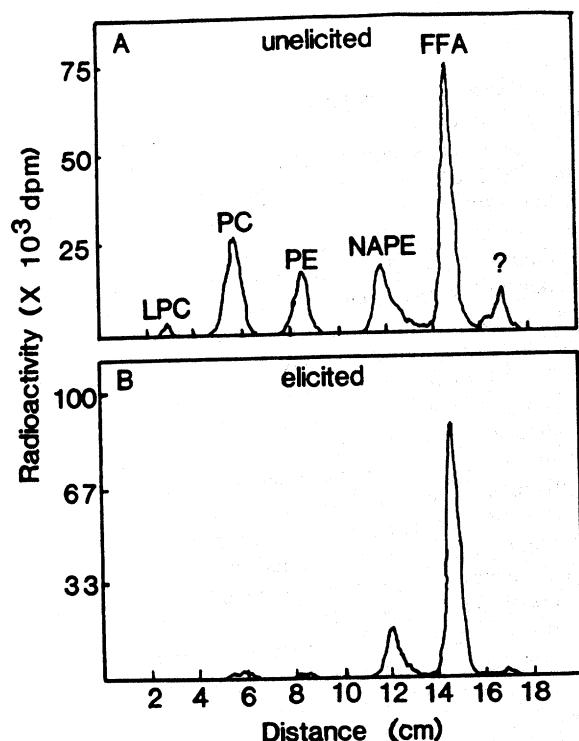


Fig. 5. Chromatograms of radioactive lipids separated by TLC following incubation of cell homogenates with [^{14}C]-palmitic acid (9.25 kBq; 2109 GBq mol $^{-1}$) for 30 min. Homogenates of unelicited cells (A) and elicitor-treated cells (B) were analyzed for incorporation of radiolabeled palmitic acid into phospholipids. Lipids were extracted from reaction mixtures as described in Materials and methods and spotted 2 cm from the bottom of the plates; direction of solvent development was to the right. Phospholipids were identified by co-chromatography with authentic standards and marked in A. Chromatograms are traces generated by radiometric scanning of TLC plates (Bioscan System 200 imaging device).

nase (Fig. 3B); this increase closely paralleled the rise and fall of NAPE levels (Fig. 3A). NAPE synthase specific activity more than doubled during the first 2 h of elicitation with xylanase (from 5.83 to 12.51 nmol [mg protein] $^{-1}$ h $^{-1}$) then dropped somewhat in the 4-h sample (to 8.23 nmol [mg protein] $^{-1}$ h $^{-1}$). Based on activities measured *in vitro*, the NAPE synthase activity was more than sufficient to account for the increased amount of NAPE observed in elicited cells.

The biosynthesis of [^{14}C]-NAPE from [^{14}C]-ethanolamine was examined in elicited and unelicited cells. A greater relative percentage of [^{14}C]-ethanolamine was accumulated in [^{14}C]-NAPE in cells treated with xylanase than in those not treated with xylanase (Fig. 4). Total radioactivity incorporated into phospholipids (almost exclusively PE and NAPE) was not significantly different in elicited vs unelicited cells. Quantitatively the amount of radioactivity in NAPE was 2.9-fold higher in elicited compared to that in unelicited cells (3 393 dpm vs 1 170 dpm).

The pattern of incorporation of exogenously supplied

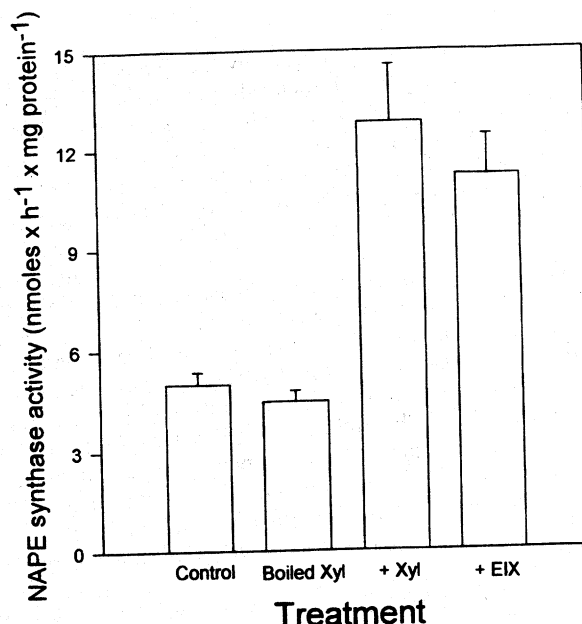


Fig. 6. Comparison of NAPE synthase specific activity in homogenates prepared from tobacco cells treated for 2 h with sterile water (Control), boiled xylanase (Boiled Xyl, 1 mg [ml cells] $^{-1}$), commercial xylanase (+ Xyl, 1 mg [ml cells] $^{-1}$), or ethylene biosynthesis-inducing xylanase (+ EIX, 1 mg [ml cells] $^{-1}$). Values presented are means and SD of measurements from three separate elicitation experiments.

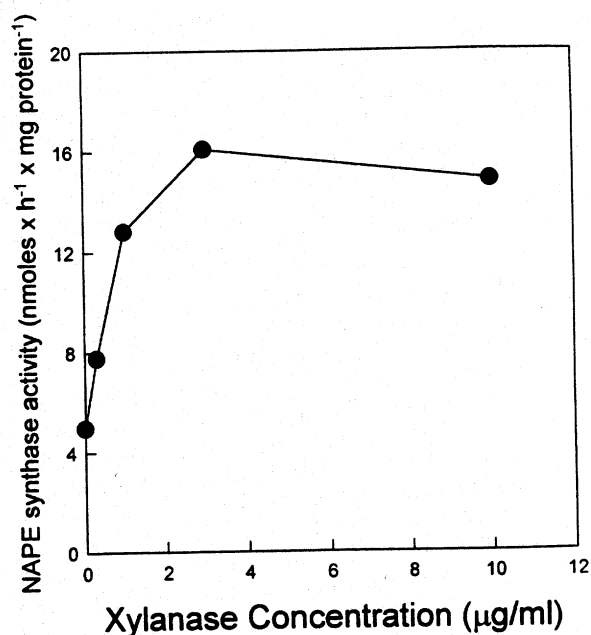


Fig. 7. NAPE synthase specific activity measurements in homogenates of tobacco cells treated for 2 h with the xylanase (commercial) concentrations of 0, 0.3, 1.0, 3.0 or 10.0 g (ml cells) $^{-1}$. Values presented are averages of duplicate samples. Similar trends were observed in two replicate experiments.

[1-¹⁴C]-palmitic acid into phospholipids by tobacco cell homogenates was dramatically influenced by elicitation (Fig. 5). In homogenates of unelicited cells, several phospholipid classes including NAPE were acylated (Fig. 5A). In homogenates of cells that had been treated with xylanase for 1 h, NAPE was almost the only phospholipid synthesized from free fatty acids (FFA) (Fig. 5B).

The degree of stimulation of NAPE synthase specific activity was similar whether cells were treated for 2 h with commercial preparations of xylanase or purified EIX (Fig. 6). Heat treatment of xylanase (100°C, 5 min) prior to its addition to cell cultures eliminated its ability to elicit an increase in NAPE synthase specific activity. A₃₈₂-absorbing material followed the same pattern (data not shown) as that shown for NAPE synthase activity; i.e. levels were induced to a similar degree in EIX and xylanase-treated cells, and heat treatment of xylanase eliminated this induction.

A range of xylanase concentrations was tested for its effect on elicitation of NAPE synthase activity (Fig. 7). Increasing concentrations of xylanase resulted in increasing levels of stimulation of NAPE synthase specific activity. Saturation with respect to NAPE synthase induction appeared to be achieved at xylanase concentrations above 3 g (ml cells)⁻¹.

Discussion

A combination of experimental evidence indicated that *N*-acylation of PE is increased during treatment of plant cells with a fungal xylanase. It is possible that similar biochemical events occur during the infection of plant cells by fungal pathogens, and these events may serve to protect the plant cells during fungal infection. Levels of NAPE and NAPE synthase specific activity were greater in elicitor-treated tobacco cells (Fig. 3). Also, biosynthesis in vivo of radiolabeled NAPE from [¹⁴C]-ethanolamine was greater in elicitor-treated cells (Fig. 4). Work with NAPE accumulation in animal tissues has suggested that NAPE plays a role in protection of biological membranes. The *N*-acyl moiety of NAPE is believed to intercalate into the hydrophobic interior of the bilayer (Akoka et al. 1988, Lafrance et al. 1990) and thus provide a membrane stabilizing effect. Hence, Schmid and coworkers (1990) have proposed that NAPE is synthesized in intracellular membranes during tissue damage to help maintain compartmentation and cell function. It is possible that NAPE is synthesized in tobacco cells in response to xylanase treatment to serve an analogous function. Future work will be aimed at elucidating the function of these unusual membrane lipid acylation events and their relationship, if any, to plant defense at the membrane level.

The mechanism of xylanase recognition by plant cells is not known; however, evidence suggested that the protein itself, and not enzymatic products, was responsible for elicitor activity (Felix et al. 1993, Sharon et al.

1993). Experiments with protoplasts indicated that xylanase probably acts directly at the level of the plasma membrane of plant cells (Sharon et al. 1993). Further, Bailey et al. (1992) demonstrated that changes in membrane function/permeability of cell suspensions and intact tissues of tobacco were elicited by EIX. EIX-treated cells exhibited increased efflux of K⁺, influx of Ca²⁺, and increased leakage of intracellular components within 2 h of treatment. Rapid changes in membrane permeability (extracellular alkalinization) also were observed in tobacco cells treated with commercially available xylanase or purified EIX (Felix et al. 1993). It is possible that changes in membrane permeability trigger an increase in NAPE biosynthesis (and acylation of membrane sterols) in tobacco cells in an effort to reduce membrane leakage and cell damage. In support of this concept, results reported by Domingo et al. (1993) indicated that increasing the NAPE content of PC liposomes reduced the permeability of model membranes to K⁺ and carboxyfluorescein. Nevertheless, the causal relationship between changes in membrane permeability induced by xylanase, the acylation of SG, and the *N*-acylation of PE remain to be determined.

Moreau and coworkers (Moreau et al. 1994) found that commercially available xylanase from *T. viride* was similar to purified EIX with respect to promoting the acylation of St and SG. Consistent with these findings, our results demonstrated that NAPE synthase specific activity was increased to similar levels after 2 h treatment with xylanase or purified EIX (Fig. 6). It is doubtful that the increased acylation of these lipids involved ethylene biosynthesis, because while EIX induced ethylene production in tobacco tissues, it inhibited the production of ethylene in cell suspensions isolated from those tissues (Bailey et al. 1992). The decrease in ethylene production by cultured cells was attributed to leakage of 1-aminocyclopropane-1-carboxylate (ACC), the precursor of ethylene, from elicitor-treated cells. Moreover, xylanase has been shown to elicit certain defense responses (e.g. synthesis of pathogenesis-related proteins; Lotan and Fluhr 1990) in tobacco by a signal-transduction pathway independent of ethylene. The results presented here will provide the basis for additional investigations as to the precise mechanism(s) responsible for increasing intracellular NAPE synthesis after exposure of plant cells to fungal elicitors.

Incorporation of FFA into phospholipids in cell-free extracts of tobacco cells was apparently modulated by elicitation. The physiological relevance of this observation is unclear; however, it may be related to the mechanisms of acylation of these lipids. *N*-acylation of PE is an ATP-independent process and utilizes unesterified fatty acids as the acyl substrate (Chapman and Moore 1993b, 1994, McAndrew et al. 1995). On the other hand, all of the other phospholipids (LPC, PC, PE) are synthesized from FFA via acyl-coenzyme A (acylCoA) intermediates and this depends upon the hydrolysis of ATP (reviewed in Frentzen 1993). The difference in phospho-

lipid acyl labeling by FFA may actually be a reflection of changes in the acylCoA pool (higher in elicited cells) or levels of ATP (lower in elicited cells). A larger endogenous acylCoA pool would result in lower labeling of the acylCoA pool by exogenously supplied [1-¹⁴C]-palmitic acid. The incorporation of [1-¹⁴C]-palmitic acid into [¹⁴C]-NAPE would be relatively unaffected. ASG is synthesized from acylCoA and therefore accumulation of ASG may be a metabolic consequence of elevated acylCoA levels. More information is needed to interpret with confidence the intriguing differences in acyl labeling in elicitor-treated cells.

Collectively, these results extend previous observations of changes in membrane lipids in elicitor-treated plant cells to include increased synthesis of NAPE. We suggest that NAPE may play a protective role in membranes of plant cells as has been suggested for the accumulation of NAPE in animal tissues (Schmid et al. 1990).

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